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Multiplex-PCR Method for Species Identification of Coagulase-Positive Staphylococci▽

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In veterinary medicine, coagulase-positive staphylococci (CoPS) other than *Staphylococcus aureus* have frequently been misidentified as being *S. aureus* strains, as they have several phenotypic traits in common. There has been no reliable method to distinguish among CoPS species in veterinary clinical laboratories. In the present study, we sequenced the thermonuclease (*nuc*) genes of staphylococcal species and devised a multiplex-PCR (M-PCR) method for species identification of CoPS by targeting the *nuc* gene locus. To evaluate sensitivity and specificity, we used this M-PCR method on 374 staphylococcal strains that had been previously identified to the species level by an *hsp60* sequencing approach. We could successfully distinguish between *S. aureus*, *S. hyicus*, *S. schleiferi*, *S. intermedius*, *S. pseudintermedius*, and *S. delphini* groups A and B. The present method was both sensitive (99.8%) and specific (100%). Our M-PCR assay will allow the routine species identification of CoPS isolates from various animal species for clinical veterinary diagnosis.

The genus *Staphylococcus* is present in skin and nasal flora and causes opportunistic infections in humans and various animals. To date, seven species of coagulase-positive staphylococci (CoPS) have been identified: *Staphylococcus aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini*, and *S. pseudintermedius* (10, 12). In addition to *S. aureus*, the other CoPS species can cause severe infections compared with those caused by coagulase-negative staphylococci (CoNS) (2, 13, 16, 22, 25, 28, 32).

It is known that staphylococcal species exhibit host specificity, and the species of CoPS isolated from clinical specimens differ with host animal species; for example, the predominant species in ruminants, pigs, dogs, and pigeons (*Columba livia*) are *S. aureus*, *S. hyicus*, *S. pseudintermedius*, and *S. intermedius*, respectively (11, 28). MIC breakpoints of oxacillin to determine methicillin resistance differ with species. Therefore, the identification of CoPS to the species level is an important task for veterinary diagnostic laboratories.

It is difficult to discriminate between CoPS species based on phenotypic differences because there is a lack of unique biochemical markers for species identification (12, 28). Although various molecular methods have been reported (1, 3, 6, 7, 19, 20, 24, 28), they are costly and/or time-consuming, and interpretation of the results is complicated. Thus, a simple and precise method for discriminating among CoPS species is highly desirable.

In the present study, we performed a sequence analysis of *nuc* genes in CoPS and related species and developed a multiplex-PCR (M-PCR) method for the species identification of the CoPS-targeted *nuc* gene locus.

MATERIALS AND METHODS

Bacterial strains and species identification. As shown in Table 1, eight CoPS strains and six closely related CoNS species were used for phylogenetic analysis based on thermonuclease (*nuc*) genes. To evaluate the sensitivity and specificity of M-PCR for the species identification of CoPS, 374 staphylococcal strains derived from various animal species were used in the present study (Table 2).

All strains used in this study were identified to the species level by sequencing analysis based on the *hsp60* gene (19). The discrimination among *S. delphini* groups A and B was performed by using a *nuc* gene sequencing method reported previously (28). The identification of *S. schleiferi* to the subspecies level was performed by a coagulase test using rabbit serum (Eiken Chemical Co., Ltd., Tokyo, Japan).

Strains were stored in 10% skim milk at −80°C until use and were maintained on Trypticase soy agar II with 5% sheep blood (BD Japan, Co., Ltd., Tokyo, Japan).

DNA extraction. A single colony was suspended to a 1.0 McFarland standard in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) with 10 U of achromopeptidase (Wako Chemical Co., Ltd.), and the suspension was incubated at 55°C for 10 min. Supernatants were used as crude DNA extracts for PCR.

Amplification and sequence analysis of the *nuc* gene. In order to amplify the conserved regions of *nuc* genes, degenerate primers were designed by multiple alignments of amino acid sequences of the staphylococcal *nuc* genes, which were available from NCBI databases. Primers Nuc-alF1 (5′-CCNAAYACNCCNGTNCARCCN-3′) and Nuc-alR (5′-NADCCANACRTANGCNARNGT-3′) were used. The reaction mixture for the PCR consisted of 2 μl of DNA extract in a total volume of 50 μl composed of 2 U of Ex *Taq* (Takara Co., Ltd., Kyoto, Japan), 30 pmol each primer, 0.2 mM deoxynucleoside triphosphate mixture, and 1× reaction buffer (Takara). Reaction mixtures were thermally cycled once at 95°C for 2 min; 30 times at 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and then once at 72°C for 2 min. The PCR product was cloned into plasmid pCR-4 I-TOPO (Invitrogen, Life Technologies, Carlsbad, CA) and was transformed into *Escherichia coli* TOP10 cells (Invitrogen). Insert DNA of the recombinant plasmid was sequenced by using a Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) with an ABI Prism 3100

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TABLE 1. Strains used for sequencing analysis of the thermonuclease (*nuc*) gene

Species	Strain	Source	Size of ORF (bp)	GenBank accession no. for sequence of *nuc*	Reference or source
S. aureus	N315	Human	534	BA000018	18
S. pseudintermedius	LMG 22219T	Cat	507	AB327164	28
S. delphini group A	LMG 22190T	Dolphin	516	AB327167	This study
	CCUG 51769	Mink	507	AB327168	This study
S. delphini group B	P-27B	Pigeon	507	AB327166	28
S. intermedius	ATCC 29663T	Pigeon	507	AB327165	28
S. schleiferi subsp. *schleiferi*	P-45C	Pigeon	504	None	This study
S. schleiferi subsp. *coagulans*	JCM 7470T	Dog	504	AB465334	This study
S. hyicus	JCM 2423T	Pig	510	AB465332	This study
S. felis	JCM 7469T	Cat	507	AB465335	This study
S. chromogenes	P-29B	Pigeon	507	AB465333	This study
S. haemolyticus	JCSC 1435	Human	537	AP006716	29
S. epidermidis	RP62A	Human	537	CP000029	15
S. saprophyticus	ATCC 15305T	Human	531	AP008934	17

genetic analyzer (Applied Biosystems). The 5′ and 3′ regions were obtained by inverse PCR, and the complete *nuc* gene sequences were determined. All staphylococcal species sequenced in previous studies harbored the *nuc* gene at a specific gene locus (*nuc* gene locus), which was located about 2 to 8 kbp downstream of the aspartate kinase gene (SA1163) (15, 17, 18, 29). For the species for which degenerate PCR of the *nuc* gene was inadequate, degenerate primers targeting the aspartate kinase gene were designed. Primers Nuc-AsdF (5′-WRNCKRTTCATNARRTAYTT-3′) and AsdR (5′-ACNTAYMGNGARATGMGNGAR-3′) were used to amplify the conserved regions of the aspartate kinase genes. Reaction mixtures were thermally cycled once at 95°C for 2 min; 30 times at 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and then once at 72°C for 2 min. Downstream sequences were analyzed by inverse PCR in order to determine the complete sequence of *nuc*. Multiple alignment was carried out by using the CLUSTAL W program (30). Construction of the phylogenetic tree was performed by the neighbor-joining method (26).

M-PCR for species identification of CoPS. Primers for M-PCR were designed to amplify a portion of the *nuc* gene locus (Table 3). The reaction mixture for PCR consisted of 2 μl of DNA extract in a total volume of 50 μl composed of 2 U of Ex *Taq* (Takara Co., Ltd., Kyoto, Japan), 10 pmol each primer, 0.2 mM deoxynucleoside triphosphate mixture, and 1× reaction buffer (Takara). Reaction mixtures were thermally cycled once at 95°C for 2 min; 30 times at 95°C for 30 s, 56°C for 35 s, and 72°C for 1 min; and then once at 72°C for 2 min. DNA fragments were analyzed by electrophoresis in 1× Tris-acetate-EDTA on a 1% agarose gel stained with ethidium bromide.

In order to evaluate sensitivity and specificity, we applied the present M-PCR method to 314 CoPS and 60 CoNS strains (Table 2).

RESULTS

Sequence analysis of *nuc* genes of staphylococci. As shown in Table 1, all staphylococcal species analyzed in this study had kept the *nuc* gene. With regard to the *nuc* phylogenetic tree (Fig. 1), the relationship among CoPS species other than *S. delphini* group B agreed with that determined by the 16S rRNA and *hsp60* genes (14, 19). The *nuc* sequence for *S. delphini* group B was more closely related to that of *S. pseudintermedius* LMG 22219^T than to that of *S. delphini* LMG 22190^T (*S. delphini* group A), as previously reported (28). Five *S. schleiferi* subsp. *coagulans* strains were phylogenetically indistinguishable from 31 *S. schleiferi* subsp. *schleiferi* strains (data not shown).

The nucleotide identity of the *nuc* genes among the CoPS and closely related CoNS species ranged from 60.0 to 95.9% (mean, 71.7%). The most similar pair was *S. pseudintermedius* and *S. delphini* group B (95.9%).

M-PCR targeting the *nuc* gene locus for species identification of CoPS. By using this M-PCR method, seven species of CoPS (*S. aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. delphini* group A, *S. hyicus*, *S. pseudintermedius*, and *S. delphini* group B) showed a successful amplification of internal fragments with the expected sizes (359 bp, 430 bp, 526 bp, 661 bp, 793 bp, 926 bp, and 1,135 bp, respectively) with the primer pairs specific for each species (Fig. 2). Among the tested CoPS strains, all strains other than an *S. pseudintermedius* strain were correctly identified to the species level. This *S. pseudintermedius* strain had a 1.6-kbp insertion mutation (78% nucleotide identity with a partial sequence of the IS*1181* transposase) within the *nuc* gene open reading frame (ORF). In addition to *S. schleiferi* subsp. *coagulans* strains, all *S. schleiferi* subsp. *schleiferi* strains had 526-bp fragments amplified by the M-PCR method. Consequently, our method had no discriminating power at the subspecies level for *S. schleiferi*. We also applied this method to *S. lutrae* CCUG 38494^T and 29 CoNS species other than *S. schleiferi* subsp. *schleiferi*, and no false-positive result was observed. Consequently, this method is both sensitive (99.8%) and specific (100%).

DISCUSSION

The species identification of CoPS needs to be performed accurately in veterinary clinical laboratories for two reasons.

The first reason is that the MIC breakpoints of oxacillin to determine methicillin resistance in staphylococci differ with species. According to Clinical and Laboratory Standards Institute (CLSI) guidelines, MIC cutoff values for oxacillin for determining methicillin resistance against *S. aureus* and *S. lugdunensis* are 4 μg/ml and differ from the values for other species (0.5 μg/ml) (8). It was previously reported that some strains exhibited oxacillin MICs of 0.5 to <4 μg/ml among *mecA*-positive *S. pseudintermedius* strains (4, 9, 27). If such strains are not identified as *S. pseudintermedius* strains but are identified as *S. aureus* strains, they could be misidentified as being methicillin-susceptible strains. Such inadequate species identification could lead to suboptimal or inappropriate treat-

TABLE 2. Staphylococcal strains used as the "gold standard" population previously identified to the species level by an *hsp60* and/or *nuc* sequencing method

Species	Total no. of isolates	Host origin(s) (no. of isolates)
CoPS		
S. aureus	133	Human (33), chicken (17), pig (10), horse (5), cat (44), dog (5), bovine (19)
S. intermedius	12	Pigeon (12)
S. schleiferi subsp. *coagulans*	5	Dog (3), cat (1), polar bear (1)
S. delphini group A	17	Pigeon (12), horse (3), mink (1), dolphin (1)
S. delphini group B	8	Pigeon (2), horse (6)
S. hyicus	27	Pig (21), chicken (6)
S. pseudintermedius	111	Dog (97), cat (12), human (2)
S. lutrae	1	Otter (1)
CoNS		
S. schleiferi subsp. *schleiferi*	31	Pigeon (16), dog (12), cat (3)
S. felis	1	Cat (1)
S. chromogenes	1	Pig (1)
S. muscae	1	Pig (1)
S. sciuri	1	Cat (1)
S. vitulinus	1	Horse (1)
S. fleurettii	1	Chicken (1)
S. lentus	1	Mouse (1)
S. simulans	1	Dog (1)
S. piscifermentans	1	Pig (1)
S. pettenkoferi	1	Cat (1)
S. auricularis	1	Pig (1)
S. equorum	1	Horse (1)
S. xylosus	1	Horse (1)
S. saprophyticus	1	Human (1)
S. nepalensis	1	Mouse (1)
S. kloosii	1	Chicken (1)
S. gallinarum	1	Chicken (1)
S. succinus	1	Horse (1)
S. arlettae	1	Horse (1)
S. cohnii	1	Pig (1)
S. haemolyticus	1	Human (1)
S. pasteuri	1	Common marmoset (1)
S. warneri	1	Human (1)
S. hominis	1	Human (1)
S. lugdunensis	1	Human (1)
S. capitis	1	Human (1)
S. caprae	1	Human (1)
S. simiae	1	Common marmoset (1)
S. epidermidis	1	Human (1)

ment decisions for methicillin-resistant staphylococcal infections (5, 23, 27).

The second reason is the public health issue of whether methicillin-resistant staphylococcal isolates from pet and farm animals are *S. aureus* strains. The isolation of methicillin-resistant *S. aureus* (MRSA) strains is also now increasingly common in veterinary medicine. There are significant concerns about the potential for household pets, horses, and food-producing animals to act as reservoirs of MRSA, with subsequent transmission to humans (21, 31). Therefore, a precise diagnosis of MRSA colonization or infection in animals is a necessary social mission for clinical veterinarians. Our M-PCR method will allow the routine identification of CoPS isolates from various animal species in veterinary clinical laboratories and will provide important clues for approaching the issue.

TABLE 3. Oligonucleotide primers for M-PCR for species identification of coagulase-positive staphylococci and *S. schleiferi* subsp. *schleiferi*

Primer	Sequence (5′–3′)	Size of PCR product (bp)	Species
au-F3	TCGCTTGCTATGATTGTGG	359	*S. aureus*
au-nucR	GCCAATGTTCTACCATAGC		
in-F	CATGTCATATTATTGCGAATGA	430	*S. intermedius*
in-R3	AGGACCATCACCATTGACATATTGAAACC		
sch-F	AATGGCTACAATGATAATCACTAA	526	*S. schleiferi* subsp. *coagulans*
sch-R	CATATCTGTCTTTCGGCGCG		*S. schleiferi* subsp. *schleiferi*
dea-F	TGAAGGCATATTGTAGAACAA	661	*S. delphini* group A
dea-R	CGRTACTTTTCGTTAGGTCG		
hy-F1	CATTATATGATTTGAACGTG	793	*S. hyicus*
hy-R1	GAATCAATATCGTAAAGTTGC		
pse-F2	TRGGCAGTAGGATTCGTTAA	926	*S. pseudintermedius*
pse-R5	CTTTTGTGCTYCMTTTTGG		
deb-F	GGAAGRTTCGTTTTTCCTAGAC	1,135	*S. delphini* group B
deb-R4	TATGCGATTCAAGAACTGA		

Ghebremedhin et al. previously reported a comparative analysis of interspecific similarity values of 16S rRNA, *hsp60*, *rpoB*, *sodA*, *tuf*, and *gap* gene sequences in staphylococci (90 to 99%, 74 to 93%, 71.6 to 93.6%, 81.5 to 98%, 86 to 97%, and 24 to 96%, respectively) (14), which indicate the ranges of nucle-

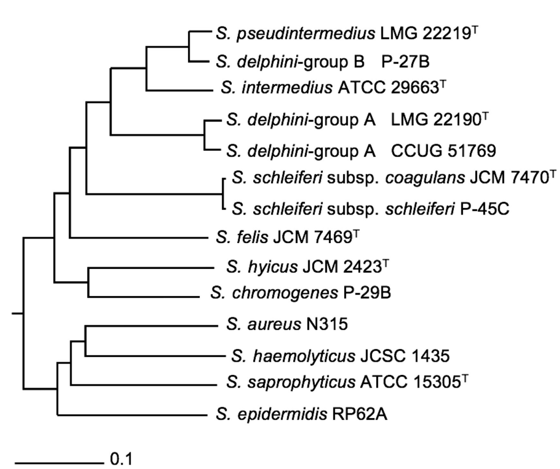

FIG. 1. Phylogenetic tree based on complete thermonuclease (*nuc*) gene sequences in staphylococci. The tree was constructed by the neighbor-joining method using CLUSTAL W.

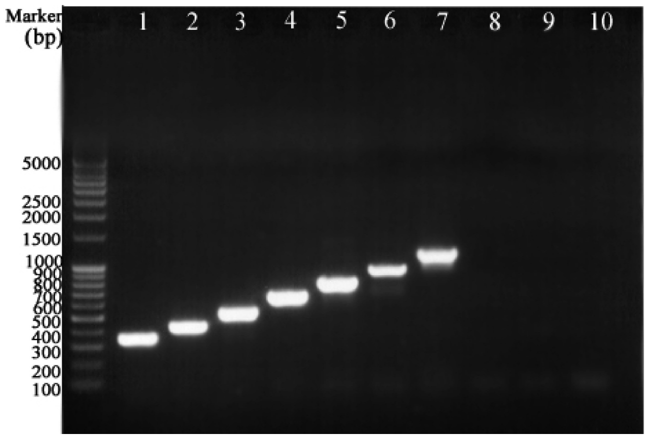

FIG. 2. Electrophoresis after multiplex PCR for species identification of coagulase-positive staphylococci (CoPS) on a 1.0% agarose gel. Lanes: 1, *S. aureus*; 2, *S. intermedius*; 3, *S. schleiferi*; 4, *S. delphini* group A; 5, *S. hyicus*; 6, *S. pseudintermedius*; 7, *S. delphini* group B; 8, *S. lutrae*; 9, *S. felis*; 10, water (negative control).

otide identity scores. The *nuc* gene has been well conserved and has shown moderate diversity among members of the genus *Staphylococcus*. Therefore, we considered this gene to be a suitable PCR target for species identification.

To date, there has been no reliable method to distinguish among CoPS species in veterinary clinical laboratories. Recently, Bannoehr et al. and Blaiotta et al. reported molecular identification methods for CoPS species by PCR-restriction fragment length polymorphism (PCR-RFLP) targeting the partial *pta* (encoding phosphotransacetylase) and *katA* (encoding catalase) genes, respectively (3, 7). Compared to these approaches, the present M-PCR method is excellent in terms of rapidity, simplicity, and cost and is better suited for clinical veterinary applications.

We previously reported that phenotypically identified *S. intermedius* strains were reclassified as being *S. intermedius*, *S. pseudintermedius*, and *S. delphini* strains by DNA-DNA hybridization and phylogenetic analysis based on partial *sodA* and *hsp60* gene sequences. In addition, *S. delphini* strains were divided into two clusters (*S. delphini* groups A and B) by *nuc* sequencing analysis (28). Although *S. delphini* group B strains were more closely related to *S. pseudintermedius* strains than to *S. delphini* group A strains (belonging to *S. delphini* LMG 22190^{T}) upon *nuc* phylogenetic analysis (28), Blaiotta et al. recently reported that *S. delphini* group B strains (strains h-2C and P-27B) were more closely related to *S. intermedius* than to *S. delphini* group A strains according to *katA* gene analysis (7). *S. delphini* group B strains may therefore represent a unique evolutionary path among staphylococci.

In conclusion, we developed a single-PCR method for the species identification of CoPS, the sensitivity and specificity of which were confirmed by using other molecular-based methods such as *hsp60* and *nuc* sequencing approaches. The present method will contribute to future clinical and research findings for staphylococcal infections in veterinary medicine.

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